



Interference of a short-term exposure to nitrogen dioxide with allergic airways responses to allergenic challenges in BALB/c mice

Barbara Proust, Ghislaine Lacroix, Franck Robidel, Maryse Marliere, Anthony Lecomte, B. Boris. Vargaftig

► To cite this version:

Barbara Proust, Ghislaine Lacroix, Franck Robidel, Maryse Marliere, Anthony Lecomte, et al.. Interference of a short-term exposure to nitrogen dioxide with allergic airways responses to allergenic challenges in BALB/c mice. *Mediators of Inflammation*, 2002, 11 (4), pp.251-260. 10.1080/096293502900000113 . ineris-00961866

HAL Id: ineris-00961866

<https://hal-ineris.archives-ouvertes.fr/ineris-00961866>

Submitted on 20 Mar 2014

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Interference of a short-term exposure to nitrogen dioxide with allergic airways responses to allergenic challenges in BALB/c mice.

Barbara PROUST^{*†}, Ghislaine LACROIX[†], Franck ROBIDEL[†],
Maryse MARLIERE[†], Anthony LECOMTE[†], and B. Boris VARGAFTIG^{*}

**Unité de Pharmacologie Cellulaire, Unité Associée Institut Pasteur-INSERM, U485, Paris,
and [†]Institut National de l'Environnement Industriel et des Risques (INERIS), Verneuil-en-
Halatte, France*

Correspondence and requests for reprints should be addressed to:

Barbara PROUST,

Unité de Pharmacologie Cellulaire, Unité Associée-Institut Pasteur-INSERM, U485, 25 rue
du Docteur Roux, 75724 Paris Cedex 15, France.

Phone (33) 1 45 68 86 93

Fax (33) 1 45 68 87 03

E-mail:bproust@pasteur.fr

Abbreviated form of the title: Effects of inhaled NO₂ on allergic mice

Subject category: Respiratory Toxicology

Abstract

Nitrogen dioxide (NO₂) is a common indoor and outdoor air pollutant whose role in the induction of asthma is unclear. We investigated the effects of NO₂ on the development of asthma-like responses to allergenic challenge in BALB/c mice. Ovalbumin (OVA)-immunized mice were intranasally challenged with OVA or saline solution just before starting a 3 h exposure to 5 or 20 ppm NO₂ or air. 20 ppm NO₂ induced a significant increase of bronchopulmonary hyperreactivity in OVA-challenged mice and of permeability according to the fibronectin content of the bronchoalveolar lavage fluid (BALF) 24 h after exposure, as compared to air or 5 ppm. Eosinophilia (cell counts in the BALF and eosinophil peroxidase of lung tissue) was detected at 24 and 72 h with similar levels for air and 20 ppm NO₂ whereas, unexpectedly, a marked reduction was observed for 5 ppm. At 24 h, IL-5 in the BALF was markedly reduced at 5 ppm compared to 20 ppm NO₂ and was also more intense for 20 ppm than for the air group. In contrast to specific IgG1 titers, anti-OVA IgE titers and IL-4 in the BALF were not affected by NO₂ exposure. Irrespective of the concentration of NO₂, OVA-challenged mice did not develop late mucosal metaplasia compared to those exposed to OVA-air. These results indicate that a short exposure to NO₂ can exacerbate or inhibit some features of the development of allergic disease in mice and may depend to concentration of pollutant.

Keywords: Mouse model of asthma; nitrogen dioxide; air pollutant; bronchopulmonary hyperreactivity; lung permeability; eosinophilia; mucus.

Abbreviations

Bovine serum albumin, BSA

Bronchoalveolar lavage fluid, BALF

Bronchopulmonary hyperreactivity, BHR

Enzyme-linked immunosorbent assay, ELISA

Eosinophil peroxidase, EPO

Intranasally, i.n.

Interleukin, IL

Methacholine, Mch

Optical density, OD

Ovalbumin, OVA

Phosphate-buffered saline, PBS

Room temperature, RT

Introduction

Asthma is an allergic respiratory disease that has captured a great deal of attention for several years. One of its perplexing aspects is that its prevalence has increased steadily during this century, doubling in the last 20 years in most industrialized countries (Hartert *et al.*, 2000). Although asthma is familial and genome-wide searches have identified genetic loci predisposing to the disease, it is unlikely that the genetic makeup of stable populations can change significantly in less than one century. The probable cause of the epidemic must therefore relate to the environment (Hartert *et al.*, 2000). Several recent studies have shown an association between air pollution during episodes of smog and asthma exacerbations, and hospital visits for asthma (Anderson *et al.*, 1998; Morgan *et al.*, 1998; Sheppard *et al.*, 1999). Although this finding does not address causality, it supports that air pollution has an effect on acute asthmatic episodes. Current evidence also suggests that asthmatics are more sensitive to the effects of air pollutants (Koren, 1995).

Asthma is characterized by acute bronchoconstriction, late bronchopulmonary hyperreactivity, pulmonary eosinophilic inflammation, excessive mucus production and increased serum IgE titers (Djukanovic *et al.*, 1990), induced by a variety of stimuli. Its pathology seems to be directly linked to the presence in the airways of eosinophils and of Th2 CD4⁺ lymphocytes (Nakajima *et al.*, 1992; Robinson *et al.*, 1992), which produce IL-4 and IL-5. These Th2 cytokines are said to play a central role in the initiation and perpetuation of asthma (Bochner *et al.*, 1994). IL-5 regulates the growth, differentiation and activation of eosinophils and provides an essential signal for their recruitment to the lungs during allergic inflammation (Lopez *et al.*, 1988). IL-4 induces the differential development of Th0 into Th2 cells (Abelsira *et al.*, 1992), and stimulates B lymphocytes to produce IgE (Paul, 1991).

Nitrogen dioxide (NO₂), a major potent oxidant pollutant, is a well-known airway irritant (American Thoracic Society, 1996). In contrast to other pollutants, NO₂ is a widespread contaminant of outdoor and of indoor environment. Its indoor levels can exceed those found outdoor and are provided by gas cooking appliances and tobacco smoke (Goldstein *et al.*, 1988; Norman *et al.*, 1965). By contrast, the main sources of NO₂ in outdoor air are motor vehicle emissions and fossil-fuel burning industries (American Thoracic Society, 1996). Acute exposures to high concentrations of NO₂ produce changes in pulmonary function, increase airway responsiveness (Mohsenin *et al.* 1987), and induce pulmonary edema (Hajela *et al.*, 1990). NO₂ also may cause release of inflammatory mediators, and induce mast cell and lymphocyte infiltration (Sandstrom *et al.*, 1991). Animal studies have demonstrated that exposure to NO₂ can increase susceptibility to infection, presumably through its effect on lung defense mechanisms, mucociliary clearance (Hubbard *et al.*, 1994), and alveolar macrophage function (Davis *et al.*, 1992; Robison *et al.*, 1993). NO₂ is tissue-soluble, unsaturated bonds in membrane lipids of the airway and respiratory epithelium, as well lining fluid being its prime targets (Postlethwait *et al.*, 1994). Several studies have associated morbidity of asthma with elevated concentrations of NO₂ (Ostro *et al.*, 1994; Tunnicliffe *et al.*, 1994). Based on epidemiological studies, it has been suggested that NO₂ increases the risk for exacerbations of asthma (Castellsague *et al.*, 1995; Rossi *et al.*, 1993). Clinical observations have demonstrated that exposure to NO₂ correlates with exacerbations of asthma and with the potentiation of airway reactivity in asthmatics (Strand *et al.*, 1997; Strand *et al.*, 1996), in contrast to other studies, which have failed to show an association between exposure to NO₂ and increased incidence of asthma (Samet *et al.*, 1993). These discrepancies show that the role of NO₂ for allergic disease is still unclear.

Since NO₂ can increase the bronchial responsiveness to non-specific stimuli such as histamine and methacholine, it may hypothetically affect bronchial responsiveness and other features of asthma to inhaled allergen as well. The existence of such an interaction between NO₂ and allergen resulting in an augmented allergic reaction has been suggested (Molfino *et al.*, 1991; Strand *et al.*, 1997; Tunnicliffe *et al.*, 1994). In this study, we investigated the role of NO₂ in the exacerbation of asthma in an ovalbumin-immunized mouse model. For that, we studied whether a short-term exposure to low or high concentrations of NO₂ can potentiate the responses to allergenic challenge in OVA-immunized mice, in order to define its possible adjuvant role in the development of some features of asthma, such as bronchopulmonary responsiveness, eosinophilic pulmonary inflammation, production of Th2 cytokines, and of specific IgE and mucus secretion.

Materials and Methods

Animals

Male strain BALB/c mice (6-7 weeks of age, 23 ± 2 g body weight) purchased from the Centre d'Elevage R. Janvier (Le Genest Saint-Isle, France) were housed (10 mice per cage) in the INERIS animal-care unit, a facility accredited by the Departmental Direction of Veterinary Services. The animals were maintained on a 12 h light/dark cycle, at 20-24°C with relative humidity of 40-70 %, and had free access to conventional laboratory feed and water. Animals were handled in accordance with French State Council guidelines for the care and use of laboratory animals (Decree n°87-849, October the 19th 1987), and was approved by the Institutional Animal Care and Use Committee at the INERIS.

Antigen immunization and challenge

BALB/c mice were immunized by the subcutaneous injection of 10 µg ovalbumin (OVA, ICN Biomedicals, Inc., Aurora, OH), dispersed in 1.6 mg Al(OH)₃ (Merk, Darmstadt, Germany) in 0.4 ml of 0.9 % NaCl (saline) at Days 0 and 7. At Day 14, one week after the second injection, immunized mice were intranasally (i.n.) challenged with 10 µg OVA in 50 µl of saline for about 10 s under anesthesia by intravenous injection of ketamine (Imalgene®1000, 35 mg/kg, Merial, France). Control mice were challenged with the same volume of saline solution.

Exposure system

The whole body exposure system used to generate NO₂ and expose the animals was developed in the laboratory of INERIS. Unrestrained, and conscious mice challenged with OVA or saline were individually placed in a whole body glass chamber of 0.5 l and were

exposed to 5 or 20 ppm NO₂ or to air for 3 h. In each glass chamber, NO₂ was delivered with a flow rate of 5 l/min allowing to have a renewal, and was calibrated at the exact desired concentration in synthetic air. The airflow of NO₂ in each chamber was monitored by a mass flow-meter during all the period of exposure. The relative pressure of the glass exposure chamber was controlled by a manometer. Concentrations of 5 or 20 ppm of NO₂ were obtained from cylinders of NO₂ gas prepared and certified by the supplier (Air Liquide, France).

Evaluation of bronchopulmonary hyperreactivity

BHR was evaluated with a barometric plethysmography method. Unrestrained, conscious mice were placed in a whole body plethysmographic chamber (EMKA Technologies, Paris, France) which measured the respiratory waveforms. Animals were exposed to an aerosol of methacholine (Mch, Aldrich, Milwaukee, WI) for 20 s at 0.1 M delivered by an aerosolator. The index of airway obstruction was expressed as enhanced pause (Penh), in response to inhaled Mch (Hamelmann *et al.*, 1997). Penh was calculated as : $Penh = [Te \text{ (expiratory time)}/Tr \text{ (relaxation time)}-1] \times [Pef \text{ (peak expiratory flow)}/Pif \text{ (peak inspiratory flow)}]$. For the graphic representation, each value was expressed every minute and was calculated from the average of three values of Penh recorded every 20 s. In order to simplify the interpretations, the area under the curve (A.U.C) was calculated for 15 min. The graphics in terms of A.U.C represent the quantitative expression of BHR.

Serum sample preparations and bronchoalveolar lavage fluid (BALF)

Mice were anesthetized by the intraperitoneal injection of urethane (ethylcarbamate, 2 g/kg, Sigma, St Louis, MO) and the abdominal cavity was opened. Blood samples were collected from the post vena cava and serum was collected after centrifugation at 500 × g for

10 min and stored at -20°C until used. After exsanguination, the lungs were flushed via the cannulated trachea with 4 × 0.5 ml of a sterile PBS solution (phosphate buffer 10 mM, pH 7.4). The total cell numbers were counted automatically (Coulter Counter ZM, Coultronics, Margency, France). BALF was cytocentrifuged for 10 min (Cytospin, Shandon, England). Slides were stained with a May Grünwald Giemsa-derived method (Diff Quick, Baxter Dade AG, Duedingen, Switzerland), and a total of 200 cells was counted for each sample by light microscopy, the percentage of each cell population being calculated. The BALF was centrifuged for 10 min at 1850 × g, 4°C (Jouan, Saint Herblain, France) and the supernatants were removed and stored at -20°C until used.

Evaluation of lung eosinophil peroxidase activity

To quantify the lung sequestration of eosinophils, eosinophil peroxidase (EPO) activity in the lung was evaluated in 96-well plates by a cytochemical enzyme assay (Van Oosterhooft *et al.*, 1995). Briefly, lungs were removed and homogenized (Potter-Elvehjem glass homogenizer, Thomas, Philadelphia, PA) in 0.05 M Tris-HCl buffer pH 8 containing 0.1% Triton X-100 solution. Lung homogenates were centrifuged for 15 min at 1600 × g, 4°C (Sigma, Bioblock Scientific 2K15, Illkirsh, France). EPO activity was measured in the supernatant, based on the oxidation of o-phenylenediamine (Sigma) by EPO in the presence of peroxide hydrogen. Incubations in duplicate were carried out in the absence or presence of the peroxidase inhibitor 3-amino-1,2,4-triazole (AMT, Sigma). Plates were read with an automatic microplate reader (Dinatech MR 5000, Dinatech Laboratories, Saint Cloud, France) at 490 nm and results were expressed as optical density (OD).

Evaluation of cytokines

IL-4 in the BALF was evaluated by ELISA. Briefly, 96-well plates were coated with rat anti-mouse IL-4 (BVD4-1D11, Perbio Sciences, Erembodegem-Aalst, Belgium) at 2 µg/ml diluted in 0.1 M carbonate buffer pH 8.2 and incubated overnight at 4°C. Plates were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of bovine serum albumin (BSA) overnight at 4°C. After washing, dilutions of recombinant murine IL-4 (rIL-4, Perbio Sciences) (15.6 to 1,000 pg/ml) or samples were applied overnight at 4°C. Then, biotinylated rat anti-IL-4 antibody (BVD6-24G2, Perbio Sciences) was added at 0.5 µg/ml for 2 h at 4°C. Plates were incubated with ExtrAvidin[®] peroxidase conjugate (1:2,000 to each well, Sigma) for 45 min at room temperature (RT). Plates were developed with tetramethylbenzidine substrate (TMB, Kiregaard Perry Laboratories, Maryland). The reaction was stopped with sulfuric acid 2N and the plates were read at 450 nm with a microplate reader (Dinatech MR 5000, Dinatech Laboratories). The lower limit of detection of this assay is \approx 10 pg IL-4/ml sample.

IL-5 in the BALF was quantified using an immunometric assay as described previously (Eum *et al.*, 1995). Briefly, 96-well plates were coated with 10 µg/ml of rat anti-mouse IL-5 (TRFK-4). To these, were added dilutions of recombinant IL-5 (rIL-5) standard (7.6 to 1,000 pg/ml) or of the sample was added, followed by an acetylcholinesterase-labelled rat anti-mouse IL-5 antibody (TRFK-5) at 10 Ellman units/ml. Absorbance was read at 405 nm with a microplate reader (Dinatech MR 5000; Dinatech Laboratories). The lower limit of detection of this assay is \approx 5 pg IL-5/ml sample.

Evamuation of anti-OVA specific IgE and IgG1

The specific anti-OVA specific IgE and IgG1 in the serum were measured by ELISA.

For the determination of specific IgE, 96-well plates were coated with rat anti-mouse IgE (EM 95-3) at 5 µg/ml diluted in 0.5 M carbonate buffer pH 9.6 and incubated overnight at 4°C. The next day, plates were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of BSA for 2 h at RT. After blocking, plates were washed and serum samples were added and incubated overnight at 4°C. The plates were then washed and 10 µg/ml of biotinylated OVA was added. The remaining steps were performed exactly as described for IL-4.

For the evaluation of specific IgG1, 96-well plates were coated with OVA (ICN Biomedicals) at 10 µg/ml diluted in 0.1 M carbonate buffer pH 8.2 and incubated overnight at 4°C. Plates were washed with PBS/0.1 % Tween 20 and blocked with PBS containing 1 % of BSA for 1 h at 37°C. After blocking and washing, serum samples were added and incubated for 1 h at 37°C. Plates were washed and incubated with Goat anti-mouse IgG1 alkaline phosphatase conjugated (1:2,000 to each well, Caltag Laboratories, Burlingame, CA) for 1 h at 37°C. The colorimetric reaction was initiated with p-nitrophenylphosphate (1 mg/ml) (Sigma) at 37°C.

As a positive control, serum pooled from OVA-immunized and challenged mice was used. Data from serum samples were expressed according to the absorbance of positive control serum after subtracting the buffer-only blank data from both. An index was calculated as: IgE or IgG1 index = [OD sample- OD buffer only]/[OD positive control- OD buffer only].

Evaluation of fibronectin

In order to evaluate the intensity of exudation through the airways, fibronectin in the BALF was measured by the indirect competitive ELISA method described by Rennard *et al.* (1980). Briefly, 96-wells plates were coated with murine fibronectin (Anawa, Wangen, Switzerland) at 1 µg/ml diluted in 0.02 M carbonate buffer pH 9.6 and incubated overnight at 4°C. In

another 96-wells plates, BALF samples and standard fibronectin were incubated with a polyclonal rabbit anti-fibronectin antibody (1:10,000, Anawa). The next day, plates coated with fibronectin were washed with PBS/0.1% Tween 20 and blocked with PBS containing 1% of BSA for 2 h at RT. Then, plates were rinsed with PBS/0.1% Tween 20 and 100 µl of antigen-antibody were applied to plates and incubated for 2 h at RT. After washing, the anti-fibronectin antibody that did not bind with BALF fibronectin content was detected with a biotinylated secondary anti-rabbit antibody (1:1,500, Amersham Pharmacia Biotech, France) and incubated for 2 h at RT. Plates were washed and incubated with ExtrAvidin® peroxidase conjugate (1:20,000 to each well, Sigma) for 45 min at RT. The remaining steps were performed exactly as described for IL-4. The lower limit detection of this assay is $\approx 0.078 \mu\text{g}$ fibronectin/ml sample.

Lung histology

After exsanguination, the lungs were removed and fixed by intratracheal instillation with 10 % neutral phosphate-buffer formalin. The whole lung was embedded in paraffin, sectioned at a thickness of 5 µm and stained with periodic acid Schiff (PAS) to examine mucus cells in the airway walls.

Statistical analysis

The results are expressed as mean \pm standard error of the mean (SEM). Statistical comparisons between groups were made using ANOVA. Multiple comparisons between all groups were performed by Fisher's least-significant difference test. A value of $P \leq 0.05$ was considered to be significant.

Results

In order to investigate the influence of NO₂ on the responses to the i.n. administration of OVA in immunized mice, two concentrations of NO₂ that are relatively high as compared to those encountered in the environment were used, since the actual amounts delivered to the lung are quite below the concentrations inhaled (Hatch *et al.*, 1994; Kleeberger *et al.*, 1997). We have developed a murine model of allergen-induced pulmonary inflammation sharing the essential features of asthma, in which the two time points of 24 and 72 h after allergenic challenge allowed to evaluate the parameters of the asthma phenotype. One week after the booster injection of antigen, OVA-immunized mice were challenged i.n. with 10 µg of OVA or saline just before the exposure to air or to NO₂ at 5 or 20 ppm for 3 h. The time points of 24 and 72 h were studied after NO₂ exposure. The peak of BHR, the initial phase of pulmonary eosinophil entrapment, and the peak of production of Th2 cytokines were studied at 24 h, whereas at 72 h, the increase of pulmonary eosinophilic inflammation, the late mucosal metaplasia development and specific immunoglobulins in serum were determined.

Bronchopulmonary responsiveness to methacholine

At Day 15, i.e., 24 h after the exposure to 20 ppm NO₂ or to air, immunized BALB/c mice challenged with OVA expressed significant BHR as compared to those exposed to 5 ppm NO₂ (Figure 1). Mice challenged with saline exposed to air or to NO₂ did not develop BHR. In OVA-challenged mice exposed to 20 ppm NO₂, BHR was significantly increased as compared to those exposed to air and to 5 ppm. At Day 17, 72 h after exposure, OVA-challenged mice exposed to air, or to NO₂ failed to develop BHR (Figure 1).

Fibronectin exudation into the BALF

To evaluate the exudation through the airways, the concentration of fibronectin was measured in the BALF. OVA-challenged mice exposed to air or to 20 ppm NO₂ released significantly increased amounts of fibronectin in the BALF, whereas those levels were very low in animals exposed to 5 ppm NO₂ (Figure 2). In contrast, exposure to 20 ppm NO₂ augmented by 6-7 fold the fibronectin concentrations in the BALF of OVA-challenged mice after 24 h, as compared to the OVA-air group. NO₂ at 20 ppm also induced a marked fibronectin exudation in saline-challenged mice, as compared to the saline-air or saline-NO₂ 5 ppm groups. Seventy two hours after exposure, BALF fibronectin content persisted in saline- or OVA- challenged mice exposed to 20 ppm NO₂. At this time point, in OVA-challenged mice exposed to 5 ppm NO₂ or to air, the release of fibronectin was delayed, as compared to mice exposed to 20 ppm NO₂ (Figure 2). Indeed, 24 h after exposure to air or to 5 ppm NO₂, no or low fibronectin was detected in the BALF of OVA-challenged mice. But, 72 h after exposure, increased amounts of fibronectin were found, contrary to mice exposed to 20 ppm NO₂, in which exudation started at 24 h.

BALF cell infiltration and lung sequestration of eosinophils

At 24 h, neutrophil counts were increased in the BALF of OVA-challenged mice exposed to air or to NO₂. These counts were found significantly increased and in OVA-challenged mice exposed to 20 ppm, as compared to OVA-air mice. The increase in neutrophil counts in saline-challenged animals exposed to 20 ppm NO₂ was also significantly above that found in saline-air or saline-5 ppm NO₂ mice (Figure 3A). At 72 h, neutrophil counts were normalized in all groups of mice (Figure 3A). Eosinophils in the BALF were detected at 24 h and increased at 72 h, with similar numbers in OVA-challenged mice exposed to air or to 20 ppm NO₂, as compared to those exposed to 5 ppm, in which these counts were markedly reduced

after 24 and 72 h (Figure 3B). The EPO activity in the lung increased significantly in OVA-challenged mice exposed to air at 24 and 72 h. OVA-challenged mice exposed to 5 ppm NO₂ showed a marked reduction of EPO activity at 24 and 72 h, as compared to the OVA-air group, which correlated with the reduced eosinophil counts in the BALF. Significant reduction of EPO activity was also noted in saline-challenged mice exposed to 5 ppm NO₂, as compared to those exposed to air. This contrasts with results in mice exposed to 20 ppm NO₂ or to air, in which EPO titers were increased to similar levels (Figure 3C).

Th2 cytokine production in the BALF

OVA-challenged mice exposed to air or to NO₂ released IL-5 and IL-4 in the BALF at 24 h. In connexion with eosinophilia (cell counts and EPO in the lungs), the production of IL-5 in the BALF was significantly reduced in OVA-challenged mice 24 h after exposure to 5 ppm NO₂, as compared to those exposed to 20 ppm NO₂ or to air (Figure 4A). However, the production of IL-5 was increased 3-fold in OVA-challenged mice exposed to 20 ppm NO₂, as compared to the OVA-air group, and was augmented by 10-fold, as compared to those exposed to 5 ppm (Figure 4A). By contrast, the production of IL-4 in the BALF was increased to the same extent in the three groups 24 h after exposure (Figure 4B). No IL-4 nor IL-5 were detected in the BALF at the 72 h point (Figures 4A and 4B).

Production of IgE and IgG1 anti-OVA antibody in the serum

Saline- and OVA- challenged mice exposed to air or to NO₂ produced specific IgE and IgG1 detected in the serum 24 and 72 h later. The anti-OVA IgE titers increased to a similar extend in the three groups after 72 h (Figures 5A), and the both concentrations of NO₂ failed to affect their production. Specific IgG1 titers were markedly increased as compared to IgE, without significant differences between saline and OVA-challenged mice. Mice exposed to 5 ppm

NO₂ and challenged with saline or OVA showed a significant increase of IgG1 titers as compared to those exposed to air or to NO₂ at 20 ppm (Figure 5B).

Histological analysis

As expected, the bronchial epithelium of saline-challenged immunized mice exposed to air was mucus-free (Figure 6A), under conditions where that of immunized and OVA-challenged mice was markedly enriched in mucosal cells (Figure 6B). Saline- or OVA-challenged immunized mice exposed to NO₂ at 5 or 20 ppm did not develop mucosal metaplasia as compared to OVA-challenged mice exposed to air (Figures 6D and 6F).

Discussion

Studies in asthmatics have shown that air pollutants such as ozone (Molfino *et al.*, 1991), or NO₂ augment the allergic responses (Jenkins *et al.*, 1999; Strand *et al.*, 1998; Strand *et al.*, 1997; Tunnicliffe *et al.*, 1994). At high concentrations, NO₂ is a well-known airway irritant that can cause bronchial constriction in normal subjects (Hajela *et al.*, 1990; Mohesin, 1988), and enhance airway responsiveness to histamine (Bylin *et al.*, 1988) or methacholine (Mohesin, 1987) in asthmatic patients, which are more sensitive than healthy subjects. By contrast, few studies have addressed to the interaction between NO₂ and lung allergy in experimental animals. As shown here, a short-term exposure to NO₂ produces contrasting effects on the development of asthma-related responses in an OVA-immunized mouse model, which depend on the dose of NO₂. Thus, the high dose of 20 ppm potentiated BHR, exudation and release of IL-5 in the BALF after OVA challenge, under conditions where the low dose of 5 ppm failed to modify BHR, and reduced significantly pulmonary eosinophilic inflammation and the production of IL-5 in the BALF. Since a single exposure to NO₂ was used here, both doses were relatively high as compared to those encountered in the environment. It has been reported nevertheless that the final amounts delivered to the lungs are quite below the concentrations inhaled (Hatch *et al.* 1994; Kleeberger *et al.*, 1997).

The potentiation of BHR by 20 ppm NO₂ in allergic mice may be accounted for by an increased vascular/epithelial permeability, facilitating the allergen availability and accelerating the inflammatory process. NO₂ is a potent tissue-soluble oxidant, which can induce pulmonary edema at high concentrations. In our experiments, immunized animals treated with saline and exposed to 20 ppm NO₂ had increased titers of fibronectin in the BALF, which was used as an indicator of permeability, as compared to saline-air or to saline-

5 ppm NO₂ groups. The high concentration of NO₂ potentiated the effects of OVA, since the fibronectin BALF content was significantly increased in OVA-challenged mice exposed to 20 ppm, as compared to OVA-air or to OVA-5 ppm NO₂ groups, and correlated with the increased BHR. Even though BHR disappeared with time, the increased permeability persisted 72 h after exposure to 20 ppm, whereas a more delayed increase in exudation was observed in OVA-challenged mice exposed to air or to 5 ppm of NO₂. The low concentration of NO₂ had an effect similar to that of OVA alone. Since our mice underwent a single exposure to NO₂, it is possible that such low concentrations of NO₂ become effective upon repeated exposures. In this context, the most likely mechanism for NO₂-induced increment in bronchial sensitivity to inhaled allergens is the damage of epithelial cells mediated by its oxidative activity, which may increase the cell permeability to the allergen, thus increasing its delivered dose, as occurs in cultured human epithelial cell monolayers exposed to NO₂ over short periods (Devalia *et al.*, 1993). This would account for the augmented fibronectin titers in the BALF, after its exudation from the plasma. It is also possible that NO₂ acts as a permissive agent, by allowing other factors to exacerbate asthma or that underlying factors such as the intensity of allergy or inflammation may be a prerequisite for the expression of the detrimental effects of the gas. Finally, NO₂ can reduce the mucociliary activity of the airways *in vivo* (Helleday *et al.*, 1995) and *in vitro* (Devalia *et al.*, 1993), further enhancing the accessibility of the allergen to the epithelial cells, owing to its decreased clearance from the airways.

In our experiments, eosinophilia (cell counts and EPO lung content) and IL-5 in the BALF were significantly reduced in OVA-challenged mice exposed to 5 ppm. By contrast, 20 ppm NO₂ did not affect eosinophilia, under conditions where the production of IL-5 in the BALF was significantly increased as compared to the OVA-air group. Recently, Morris *et al.* (2001)

reported that the exposure to 0.7 ppm NO₂ reduces eosinophilic inflammation in allergic mice, but the IL-5 levels were not measured. The mechanisms of the decreased eosinophilic inflammation and IL-5 production in mice exposed to 5 ppm NO₂ are unknown. They may result from alterations in the regional deposition patterns of OVA and of NO₂ in the airways, or in absorbance, pulmonary clearance or antioxidant defenses. Indeed, NO₂ has a low solubility, and is poorly absorbed by the airway mucosa (Chitano *et al.*, 1995). It is also a very reactive molecule whose uptake in the respiratory system is extremely high (Chitano *et al.*, 1995). The use of mathematical dosimetry models suggests that the uptake of NO₂ between the trachea and the respiratory zone occurs to a similar extent, and peaks at the terminal bronchioles (Chitano *et al.*, 1995). It is also possible that the biphasic effect of 5 and 20 ppm NO₂ may result from pharmacodynamic alterations. Some studies have indeed demonstrated that NO₂ affects lung defense mechanisms, including mucociliary clearance, alveolar macrophages (AM), and the immune system (O'Neill *et al.*, 1995). The decreased OVA-induced IL-5 production and eosinophilia after an exposure to 5 ppm NO₂ may result from an alteration of AM function, including differences in antigen presentation by AM, or from a decreased expression of antigen-derived peptides on their surface, which may be hampered by exposure to NO₂. Indeed, Kineast *et al.* (1996) and Erroi *et al.* (1996) have shown that NO₂ exposure of LPS-stimulated human AM results in a functional impairment of AM. Furthermore, Robison *et al.* (1993) demonstrated that exposure rates to 0.5 ppm NO₂ for 0.5-10 days, reduces the arachidonate metabolism and superoxide production in response to external stimuli. Thus, NO₂ may reduce the capacity of AM to respond to immunologic stimuli, which might explain the decreased allergic responses in animals exposed to 5 ppm, in particular eosinophilia and production of IL-5.

Airway inflammation is accompanied by mucus secretion, which contributes to airway obstruction. In our experiments, irrespective of the concentration of NO₂, OVA-challenged mice did not develop mucosal metaplasia, in contrast to those exposed to air, which is probably related to mucus denaturation by NO₂-induced oxidation. The mucous layer forms a protective barrier of the airways against the effects of oxidants, which eliminates and/or scavenges the toxic components of NO₂ prior to their diffusion into the airway epithelium. The lipid content of the mucous layer, in particular esterified unsaturated fatty acids constitutes the primary scavenging oxidants (Cavanagh *et al.*, 1987). The latter demonstrated that the phospholipids of the mucous layer cannot offer a significant protection against inhaled NO₂ and that exposure to 40 ppm NO₂ in rats induces in lipid peroxidation correlated with the apparent lack of oxidant scavenging species in the mucous lining the airways.

In summary, a short-term exposure to NO₂ modifies the asthma-like responses to allergenic challenge in BALB/c mice with contrasting effects according to its concentration. Despite the relatively high concentrations of NO₂ used in our investigation, as compared to those encountered in the environment, this study provides new informations concerning the subtle interactions between an air pollutant and allergic disease.

Acknowledgments

This work has been supported by a grant of the French Ministry of Environment.

References

Abehsira-Amar, O., Gibert, M., Jolij, M., Theze, J., and Jankovic, D.G. (1992). IL-4 plays a dominant role in the differential development of Th0 into Th1 and Th2 cells. *J. Immunol.* **148**, 3820-3829.

American Thoracic Society (ATS), Committee of the Environmental and Occupational Health Assembly (1996). Health effects of outdoor air pollution: Part 2. *Am. J. Respir. Crit. Care Med.* **153**, 477-498.

Anderson, H.R., Ponce de Leon, A., Bland, J.M., Bower, J.S., Emberlin, J., and Strachan, D.P. (1998). Air pollution, pollens, and daily admissions in London 1987-1992. *Thorax* **53**, 842-848.

Bochner, B.S., Undem, B.J., and Lichtenstein, L.M. (1994). Immunological aspects of allergic asthma. *Annu. Rev. Immunol.* **12**, 295-335.

Bylin, G., Hedenstierna, G., Lindvall, T., and Sundin, B. (1988). Ambient nitrogen dioxide concentrations increase bronchial responsiveness in subjects with mild asthma. *Eur. Respir. J.* **1**, 606-612.

Castellsague, J., Sunyer J., Saez, M., and Anto, J.M. (1995). Short-term association between air pollution and emergency room visits for asthma in Barcelona. *Thorax* **50**, 1051-1056.

Cavanagh, D.G., and Morris, J.B. (1987). Mucus protection and airway peroxidation following nitrogen dioxide exposure in the rat. *J. Toxicol. Environ. Health* **22**, 313-328.

Chitano, P., Hosselet, J.J., Mapp, C.E., and Fabbri, L.M. (1995). Effect of oxidant air pollutants on the respiratory system insights from experimental animal research. *Eur. Respir. J.* **8**, 1357-1371.

Davis, J.K., Davison, M.K., Schoeb, T.R., and Lindsey, J.R. (1992). Decreased intrapulmonary killing of *Mycoplasma pulmonis* after short-term exposure to NO₂ is associated with damage alveolar macrophages. *Am. Rev. Respir. Dis.* **145**, 406-411.

Djukanovic, R., Roche, W.R., Wilson, J.W., Beasley, C.R., Twentyman, O.P., Howarth, P.H., and Holgate, S.T.(1990). Mucosal inflammation in asthma. *Am. Rev. Respir. Dis.* **142**, 434-457.

Devalia, J.L., Sapsford, R.J., Cundell, D.R., Rusnak, C., Campbell, A.M., and Davies, R.J. (1993). Human bronchial epithelial cell dysfunction following *in vitro* exposure to nitrogen dioxide. *Eur. Respir. J.* **6**, 1308-1316.

Erroi, A., Pagani, P., Sironi, M., and Salmona, M. (1996). *In vivo* exposure to NO₂ reduces TNF and IL-6 production by endotoxin-stimulated alveolar macrophages. *Am. J. Physiol.* **271**, L132-138.

Eum, S.Y., Zuany-Amorim, C., Lefort, J., Pretolani, M., and Vargaftig, B.B. (1997). Inhibition by the immunosuppressive agent FK-506 of antigen-induced airways eosinophilia and bronchial hyperreactivity in mice. *British. J. Pharmacol.* **120**, 130-136.

Goldstein, I.F., Lieber, K., Andrews, L.R., Kazembe, F., Foutrakis, G., Huang, P., and Hayes, C. (1988). Acute respiratory effects of short-term exposures to nitrogen dioxide. *Arch. Environ. Health* **43**, 138-142.

Hajela, R., Janigan, D.T., Landrigan, S., Boudreau S., and Sebastian, S. Fatal pulmonary edema due to nitric acid fume inhalation in three pulp-mill workers. *Chest* **97**, 487-489.

Hamelmann, E., Schwarze J., Takeda, K., Oshiba, A., Larsen, G.L., Irvin, C.G., and Gelfand, E.W. (1997). Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am. J. Respir. Crit. Care Med.* **156**, 766-775.

Hartert, T.V., and Stokes Peebles, R. (2000). Epidemiology of asthma: the year in review. *Curr. Opin. Pulm. Med.* **6**, 4-9.

Hatch, G.E., Slade, R., Harris, L.P., Mc Donnell W.F., Devlin, R.B., Koren, H.S., Costa D.L., and McKee, J. (1994). Ozone dose and effect in human and rats. A comparison using oxygen-18 labeling and bronchoalveolar lavage. *Am. J. Respir. Crit. Care Med.* **150**, 676-683.

Helleday, R., Huberman, D., Blomberg, A., Stjernberg, N., and Sandstrom, T. (1995). Nitrogen dioxide exposure impairs the frequency of the mucociliary activity in healthy subjects. *Eur. Respir. J.* **8**, 1664-1668.

Hubbard, A.K., Vetrano, K.M., and Morris, J.B. (1994). Acute NO₂ exposure alters inflammatory cell activation and particle clearance in silica-injected mice. *J. Toxicol. Environ. Health* **41**, 299-314.

Jenkins, H.S., Devalia, J.L., Mister, R.L., Bevan, A.M., Rusznak, C., and Davies, J.R. (1999). The effect of exposure to ozone and nitrogen dioxide on the airway response of atopic asthmatics to inhaled allergen. *Am. J. Respir. Crit. Care Med.* **160**, 33-39.

Kineast, K., Knorst, M., Müller-Quernheim, J., and Ferlinz, R. (1996). Modulation of IL-1 β , IL-6, IL-8, TNF- α , and TGF- β secretions by alveolar macrophages under NO₂ exposure. *Lung* **174**, 57-67.

Kleeberger, R.S., Zhang, L.Y., and Jakab, G.J. (1997). Differential susceptibility to oxidant exposure in inbred strains of mice: nitrogen dioxide versus ozone. *Inhal. Toxicol.* **9**, 601-621.

Koike, E., Kobayashi, T., and Utsunomiya, R. (2001). Effect of exposure to nitrogen dioxide on alveolar macrophage-mediated immunosuppressive activity in rats. *Toxicology letters* **121**, 135-143.

Koren, H.S. (1995). Associations between criteria air pollutants and asthma. *Environ. Health Perspect.* **103**, 235-242.

Lopez, A.F., Sanderson, C.J., Gamble, J.R., Campdell, H.D., Young, I.G., and Vadas, M.A. (1988). Recombinant human interleukin 5 is a selective activator of human eosinophil function. *J. Exp. Med.* **167**, 219-224.

Mohesin, V. (1988). Airway response to 2.0 ppm nitrogen dioxide in normal subjects. *Arch. Environ. Health* **43**, 242-246.

Mohesin, V. (1987). Airway responses to nitrogen dioxide in asthmatics subjects. *J. Toxicol. Environ. Health*. **22**, 371-380.

Molfino, N.A., Wright, S.C., Katz, I., Tarlo, S., Silverman, F., McClean, P.A., Szalai, J.P., Raizenne, M., Slutsky, A.S., and Zamel, N. (1991). Effect of low concentrations of ozone on inhaled allergen responses in asthmatic subjects. *Lancet* **338**, 199-203.

Morgan, G., Corbett, S., and Wlodarczyk, J. (1998). Air pollution and hospital admissions in Sydney, Australia, 1990 to 1994. *Am. J. Public Health* **88**, 1761-1766.

Morris, J.B., Olson, J.E., Symanowicz, P.T., Thrall, R.S., Cloutier, M.M., and Hubbard, A.K. (2001). Effect of nitrogen dioxide on ovalbumin-induced allergic airway disease in a murine model. *Am. J. Respir. Crit. Care Med.* **163**, A432 (Abstract).

Nakajima, H., Iwamoto, I., Tomoe, S., Matsumura, R., Tomioka, H., Takatsu, K., and Yoshida, S. (1992). CD4⁺ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into mouse trachea. *Am. Rev. Respir. Dis.* **146**, 374-382.

Norman, V., and Keith, C.H. (1965). Nitrogen oxides in tobacco smoke. *Nature* **205**, 915-916.

O'Neill, C.A., Van der Vliet, A., Eiserich, J.P., Last, J.A., Halliwell, B., and Cross, C.E. (1995). Oxidative damage by ozone and nitrogen dioxide: synergistic toxicity *in vivo* but no

evidence of synergistic oxidative damage in an extracellular fluid. *Biochem. Soc. Symp.* 61, 139-152.

Ostro, B.D., Lipsett M.J., Mann J.K., Wiener, M.B., and Selner, J. (1994). Indoor air pollution and asthma. Results from a panel study. *Am. J. Respir. Crit. Care Med.* **149**, 1400-1406.

Paul, W.E. (1991). Interleukin-4: a prototype immunoregulatory lymphokine. *Blood.* **77**, 1859-1870.

Postlethwait, E.M., and Bidani, A. (1994). Mechanisms of pulmonary NO₂ absorption. *Toxicology* **89**, 217-237.

Rennard, S.I., Berg, R., Martin, G.R., Foidart, J.M., and Gehron Robey, P. (1980). Enzyme-linked Immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* **104**, 205-214.

Robison, T.W., Murphy, J.K., Beyer, L.L., Richters, A., and Forman, H. (1993). Depression of stimulated arachidonate metabolism and superoxide production in rat alveolar macrophages following in vivo exposure to 0.5 ppm NO₂. *J. Toxicol. Environ. Health* **19**, 555-568.

Rossi, O.V.J., Kinnula, V.L., Tienari, J., and Huhti, E. (1993). Association of severe asthma attacks with weather, pollen, and air pollutants. *Thorax* **48**, 244-248.

Samet, J.M., Lambert, W.E., Skipper, B.J., Cushing, A.H., Hunt, W.C., Young, S.A., McLaren, L.C., Schwab, M., and Spengler, J.D. (1993). Nitrogen dioxide and respiratory illnesses in infants. *Am. Rev. Respir. Dis.* **148**, 1258-1265.

Sandstrom, T., Stjernberg N., Eklund A., Ledin, M., Bjermer, M., Kolmodin-Hedman, B., Lindstrom K., Rosenhall, L., and Angstrom, T. (1991). Inflammatory cell response in bronchoalveolar lavage fluid nitrogen dioxide exposure of healthy subjects: a dose response study. *Eur. Respir. J.* **3**, 332-339.

Sheppard, L., Levy, D., Norris, G., Larson, T.V., and Koenig, J.Q. (1999). Effects of ambient air pollution on nonelderly asthma hospital admissions in Seattle, Washington, 1987-1994. *Epidemiology* **10**, 23-30.

Strand, V., Svartengren, M., Rak, S., Barck, C., and Bylin, G. (1998). Repeated exposure to an ambient level of NO₂ enhances asthmatic response to a non symptomatic allergen dose. *Eur. Respir. J.* **12**, 6-12.

Strand, V., Rak, S., Svartengren, M., and Bylin, G. (1997). Nitrogen dioxide exposure enhances asthmatic reaction to inhaled allergen in subjects with asthma. *Am. J. Respir. Crit. Care Med.* **155**, 881-887.

Strand, V., Salomonsson, P., Lundahl, J., and Bylin, G. (1996). Immediate and delayed effects of nitrogen dioxide exposure at an ambient level on bronchial responsiveness to histamine in subjects with asthma. *Eur. Respir. J.* **9**, 733-740.

Tunnicliffe, W.S., Burge, P.S., and Ayres, J.G. (1994). Effect of domestic concentrations of nitrogen dioxide on airway responses to inhaled allergen in asthmatic patients. *Lancet* **344**, 1733-1736.

Van Oosterhout, A.J.M., Fattah, D., Van Ark, I, Hofman, G., Buckley, T.L., and Nijkamp, F.P. (1995). Eosinophil infiltration precedes development of airway hyperreactivity and mucosal exudation after intranasal administration of interleukin-5 to mice. *J. Allergy Clin. Immunol.* **96**, 104-112.

Figure legends

Figure 1. *Bronchopulmonary hyperresponsiveness is significantly augmented in immunized mice exposed to 20 ppm*

At Day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO₂ or to air for 3 h. At Day 15 (24 h) and 17 (72 h), BHR in response to Mch was evaluated. The graphic in terms of A.U.C represents the quantitative expression of BHR. Data are expressed as mean ± SEM. * $P \leq 0.05$, OVA-challenged mice compared with their saline control. # $P \leq 0.05$, OVA-Air group compared with NO₂-OVA group (5 or 20 ppm). • $P \leq 0.05$, saline-Air group compared with NO₂-saline group (5 or 20 ppm). $P \leq 0.05$, 5 ppm NO₂-OVA group compared with 20 ppm NO₂-OVA group. $\Delta P \leq 0.05$, 5 ppm NO₂-saline group compared with 20 ppm NO₂-saline group.

Figure 2. *BALF fibronectin content after short exposure to NO₂*

At Day 14, immunized mice were challenged i.n. with saline (□) or OVA (■) and were exposed to 5 or 20 ppm NO₂ or to air for 3 h. At Day 15 (24 h) and 17 (72 h) after exposure, mice were sacrificed. Fibronectin levels were evaluated in the BALF by ELISA. Data are expressed as mean ± SEM. * $P \leq 0.05$, OVA-challenged mice compared with their saline control. # $P \leq 0.05$, OVA-Air group compared with NO₂-OVA group (5 or 20 ppm). • $P \leq 0.05$, saline-Air group compared with NO₂-saline group (5 or 20 ppm). $P \leq 0.05$, 5 ppm NO₂-OVA group compared with 20 ppm NO₂-OVA group. $\Delta P \leq 0.05$, 5 ppm NO₂-saline group compared with 20 ppm NO₂-saline group.

Figure 3. BALF cell infiltration after exposures to NO₂

At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Differential BALF cell counts and EPO of the lungs were evaluated. A, neutrophils; B, eosinophils; C, eosinophil peroxidase. Data are expressed as mean ± SEM. * $P \leq 0.05$, OVA-challenged mice compared with their saline control. # $P \leq 0.05$, OVA-Air group compared with NO₂-OVA group (5 or 20 ppm). • $P \leq 0.05$, saline-Air group compared with NO₂-saline group (5 or 20 ppm). $P \leq 0.05$, 5 ppm NO₂-OVA group compared with 20 ppm NO₂-OVA group. $^{\Delta}P \leq 0.05$, 5 ppm NO₂-saline group compared with 20 ppm NO₂-saline group.

Figure 4. BALF Th2 cytokine levels after exposures to NO₂

At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. BALF IL-4 and IL-5 content were evaluated by ELISA. A, IL-5; B, IL-4. Data are expressed as mean ± SEM. * $P \leq 0.05$, OVA-challenged mice compared with their saline control. # $P \leq 0.05$, OVA-Air group compared with NO₂-OVA group (5 or 20 ppm). • $P \leq 0.05$, saline-Air group compared with NO₂-saline group (5 or 20 ppm). $P \leq 0.05$, 5 ppm NO₂-OVA group compared with 20 ppm NO₂-OVA group. $^{\Delta}P \leq 0.05$, 5 ppm NO₂-saline group compared with 20 ppm NO₂-saline group.

Figure 5. Specific anti-OVA IgE and IgG1 titers in the serum after exposures to NO₂

At Day 14, immunized mice were treated as in Figure 2 (Saline (□) or OVA (■)). Specific antibodies were evaluated in the serum by ELISA. A, IgE; B, IgG1. Data are expressed as mean ± SEM. * $P \leq 0.05$, OVA-challenged mice compared with their saline control. # $P \leq 0.05$, OVA-Air group compared with NO₂-OVA group (5 or 20 ppm). • $P \leq 0.05$, saline-Air group

compared with NO₂-saline group (5 or 20 ppm). $P \leq 0.05$, 5 ppm NO₂-OVA group compared with 20 ppm NO₂-OVA group. $^{\Delta}P \leq 0.05$, 5 ppm NO₂-saline group compared with 20 ppm NO₂-saline group.

Figure 6. PAS-stained histologic sections of lungs from allergic mice exposed to NO₂

At Day 14, immunized mice were treated as in Figure 2. Seventy hours after exposure, lungs were collected for histology. Goblet cells were stained with PAS in lung sections. A: Lung section of saline-challenged immunized mice exposed to air. B: Lung section of OVA-challenged immunized mice exposed to air. Note intense goblet cell hyperplasia (arrow). C: Lung section of saline-challenged immunized mice exposed to NO₂ at 5 ppm. D: Lung section of OVA-challenged immunized mice exposed to NO₂ at 5 ppm. Lung section of saline-challenged immunized mice exposed to NO₂ at 20 ppm. Lung section of OVA-challenged immunized mice exposed to NO₂ at 20 ppm. Note the absence of goblet cell hyperplasia in saline-challenged mice exposed to air or NO₂ in A, C and E, and in OVA-challenged mice exposed to irrespective of the concentration of NO₂ in D and F (final magnification: x 200).

*

*